CHAPTER III
EXPERIMENTAL

3.1 Materials

Styrene (S), ethylene glycol dimethacrylate (EGDMA), sorbitan monooleate (Span80), tetrahydrofuran (THF), polystyrenesulfonate (PSS, MW 70,000), poly(diallyldimethylammonium chloride) (PDADMAC, MW 350,000), gelatin (GEL, type B from bovine skin), and alginic acid (ALG) were purchased from Sigma-Aldrich Chemical. Potassium persulfate ($K_2S_2O_8$) and calcium chloride ($CaCl_2$) were purchased from Fluka Chemie, Thailand. Phosphoric acid (H_3PO_4) were purchased from merck. Hydroxyapatite (HA) was purchased from MK nano, USA and used as received.

3.2 Methodology

3.2.1 Preparation of Poly(S/EGDMA) HIPE Loaded with Hydroxyapatite

Poly(S/EGDMA) polyHIPE porous foam was prepared by using the high internal phase emulsion technique as described by Akay *et al.* (2004). The oil phase (10% of total volume) contained styrene monomer (S), ethylene glycol dimethacrylate (EGDMA) as a crosslinking agent (S: EGDMA; 4:1 ratio by volume), and sorbitan monooleate (Span80, 2 ml) as a surfactant. While the aqueous phase (90% of the total volume) contained deionized water (88 ml), tetrahydrofuran (THF, 1 ml), potassium persulphate ($K_2S_2O_8$, 0.2 g) as initiator, and calcium chloride ($CaCl_2$, 1 g) as stabilizing salt, hydroxyapatite (HA, 0.45g), and phosphoric acid (H_3PO_4, 1 ml) which is used to dissolve the hydroxyapatite. The aqueous phase was added drop-wise into the oil phase under constant mechanical stirring until all aqueous phase has been added. After emulsification, the resulting emulsion was transferred into glass vials (20 mm internal diameter) and then placed in a water bath for polymerization at 60 °C for 48 hours. After that, all the samples were washed to remove any residual materials. Washing was carried out in isopropanol and then with

water in Soxhlet extractor. All samples were dried in oven at 60 °C until a constant weight was obtained.

3.2.2 Layer-by-Layer (LbL) Surface Modification

To increase hydrophilicity of poly(S/EGDMA) HIPE by using the layer-by-layer polyelectrolyte multilayers (PEM) technique. Poly(S/EGDMA) HIPE were cut to 1.5 mm thick and 20 diameter. PEM coating was performed by injected polymer solution through poly(S/EGDMA) HIPE disk. There are two coating consisted of the primary coating and the secondary coating. For primary coating, PDADMAC solution (10 mM, 10 ml) was manually injected through polyHIPE disks as positively charged polyelectrolyte for 2 min and then follow by PSS solution (10 mM, 10 ml) as negatively charged polyelectrolyte for 2 min. After each was injected in the electrolyte, the polyHIPE disks were rinsed for 30 s three times with deionized water to remove excess polyelectrolyte. This process was repeated until seven layers of polyelectrolyte had been obtained. The secondary coating was carried out using the similar procedure but different negatively charged polyelectrolyte such as alginic acid and gelatin were used.

3.3.3 Characterization of Poly(S/EGDMA) HIPE Loaded with Hydroxyapatite

3.3.3.1 Fourier Transform Infrared Spectroscopy (FT-IR)

To confirm the existence of hydroxyapatite, PSS, alginatic acid and gelatin on surface of the polyHIPE, the Atteuated Total Reflectance(ATR) technique was used.

3.3.3.2 Scanning Electron Microscope (SEM) and EDX

To investigate the phase morphology of the polyHIPE by using Scanning electron microscopy(FE-SEM, Hitachi S-4800). The specimens were coated with platinum under vacuum before testing and viewed by using accelerating voltage of 10kV.

The SEM/EDX studies were performed generally for the material identification and dispersion. In this case, hydroxyapatite load poly(S/EGDMA)HIPE scaffold was identified. All in the glove box filled with Ar

gas (99.999%) and coupled to the SEM/EDX chamber, SEM microphotographs and EDX spectra were recorded.

3.3.3.3 Surface Area Measurement

N_2 adsorption-desorption isotherms were obtained at -196°C on a Quantachrome Autosorb-1MP. Samples were degassed at 110°C during 12 hours in a vacuum furnace prior to analysis. Surface areas were calculated using the BET equation.

3.3.3.4 Contact Angle Measurement

The static contact angle measurement was performed using a Krüss (model DSA 10) contact angle measuring instrument at ambient temperature to prove the wettability change of the layer-by-layer coated surface poly(S/EGDMA) HIPE scaffold. A 10 μL sessile droplet of de-ionized water was then vertically dropped with a micro-syringe onto the foam surface. The contact angles were using the drop shape analyzer program and were then averaged.

3.3.3.5 Mechanical Properties

Lloyd Universal testing machine was used to measure mechanical properties of all samples in compression mode, according to ASTM D822. Test specimens in a cylinder shape 2.54 cm in diameter × 2.54 cm in height were prepared. A speed of 0.127 cm/min and 500 N load cells were used for all measurements. The value of the compression stress and the Young's modulus were determined from an average of five samples.

3.3.4 Cell Culture

Mouse fibroblast connective tissue (L929) was used in this study in order to investigate the ability of the poly(S/EGDMA) polyHIPE foam to act as a scaffold in tissue engineering applications. L929 fibroblast-like cells were grown in Dulbecco' s modified Eagle' s medium (DEME: Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (FBS, BIOCHROM AG), together with 100 U ml-1 penicillin (GIBCO) and 100 μg/ml streptomycin (GIBCO). The medium was replaced every 3 days and the cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. Each poly HIPE foam scaffold was cut into circular discs (about 15 mm in diameter and 1 mm thick), which were later sterilized in an

autoclave for 1 h prior to use and then the disc specimens were placed in the wells of a 24-well tissue-culture polystyrene plate (TCPS; Biokom Systems, Poland). The specimens were pressed with a metal ring (about 12 mm in diameters) in order to prevent the polyHIPE foam specimen from floating in the culture medium, and subsequently they were immersed in 500 µl of the culture medium overnight before cell seeding. The L929 fibroblast-like cells from the culture plate were trypsinized with 0.25% trypsin containing 1 mM EDTA (GIBCO) and were counted by a hemacytometer (Hausser Scientific, USA). They were then seeded at a density of 40,00 cells/well on the polyHIPE specimens and TCPS were used as controls.

3.3.4.1 Cytotoxicity Test

Evaluation of the cytotoxicity of the poly(S/EGDMA) polyHIPE foam using L929 fibroblast-like cells was done based on the stand method (ISO 10993-5). To prepare an extracted medium, circular polyHIPE specimens were sterilized in an autoclave for 1 h and placed in a 24-well plate, then washed 3 times with a serum free medium (SFM) before further incubating at 37 °C in a fresh culture medium for 24 hours. L929 fibroblast-like cells were seeded in the wells of a 24-well plate at a density of 40,000 cells/well with serum-containing DMEM for 48 h. After that, the DMEM was removed and replaced with the poly(S/EGDMA) polyHIPE foam extraction medium before an additional 24-hour incubation period. The measurement of cell viability was done using a 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT; Sigma Aldrich, USA)assay.

3.3.4.2 Cell Culture and MTT Assay

Attachment and Proliferation

For cell attachment and proliferation was study as described by Pakeyangkoon *et al.* (2013). L929 fibroblast-like cells at a density of 40,000 cells/well were used. Circular polyHIPE foam specimens (15mm in diameter and 1 mm thick) were placed in a 24-well culture plate with a metal ring. All polyHIPE foam samples were sterilized in an autoclave for 1 h, washed two times with phosphate buffer saline (PBS) and then with the culture medium (DMEM). Before cell seeding, 500 µl of the DMEM was added to each well of the 24-well culture plates. L929 fibroblast-like cells, at a density of 40,000 cells/well, were seeded on the polyHIPE foam samples and culture plate as control at 1, 4, and 24 h for the cell

attachment study. Each time point, the cell attachment number was determined by MTT assay. The proliferation of L929 fibroblast-like cells was determined at different culture periods (4 h , 1 day , 3 days, and 7 days) then measured again with MTT assay to determine the changes in the number of viable cells. In addition, the effect of plasma surface modification and treatment time on the cell attachment of the poly(S/EGDMA) polyHIPE foam was also investigated. In this part, L929 fibroblast-like cells, at a density of 40,000 cells/well, were seeded on the polyHIPE foam and on the culture plate as control for 1 day. Determination of the amount of cell attachment was also done using MTT assay.

MTT Assay

A 3-(4,5-dimethykthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT: Sigma Aldrich, USA) assay is a quantitative method and standard colorimetric assay (an assay which measures changes in color) for the measurement of cell viability and growth. The reduction of yellow tetrazolium salt in metabolically active cells to form an insoluble purple formazan crystal product by the dehydrogenase enzymes secreted from the mitochondria of viable cells. This assay can also be used to determine the cytotoxicity of potential medicinal agents and other toxic materials. Firstly, the cell-contained polyHIPE foam washed two times with PBS to remove any unattached cells, and then a 300 µl MTT stock solution (5 mg/mL in medium without phenol red) was added to each well and incubated 37 °C for 30 min. After incubation of the cells with the MTT solution, a buffer solution containing dimethylsulfoxide (DMSO: 900 µl / well) and glycine buffer (100 µl/well) was placed in each well in order to extract the purple formazan crystal and determine their by using microplate reader at a wavelength of 570 nm.

3.3.4.3 Morphological Observation of Cell Culture

The morphology of the L929 fibroblast-like cells containing poly(S/EGDMA) polyHIPE foam was observed using a scanning electron microscope (SEM). All of the polyHIPE foam was washed twice with PBS, and then cell fixation was done with a 3% glutaraldehyde solution (diluted from 50% glutaradehyde solution with PBS) at 500 ml/well for 30 min. After the fixation, the polyHIPE foam was washed with PBS and dehydrated with ethanol solutions of varying concentration (i.e. 30, 50, 70, 90, and 100%) for about 2 min at each

concentration. After being dried completely, the specimens were mounted on copper stubs, and coated with gold to observe the cell adhesion on the polyHIPE foam by SEM.